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# Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation

**Issay Kitabayashi[1], Akihiko Yokoyama, Kimiko Shimizu and Misao Ohki**

Radiobiology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, Japan

[1]Corresponding author
e-mail: ikitabay@ncc.go.jp

**The AML1 transcription factor and the transcriptional coactivators p300 and CBP are the targets of chromosome translocations associated with acute myeloid leukemia and myelodysplastic syndrome. In the t(8;21) translocation, the *AML1* (*CBFA2*/*PEBP2αB*) gene becomes fused to the *MTG8* (*ETO*) gene. We previously found that the terminal differentiation step leading to mature neutrophils in response to granulocyte colony-stimulating factor (G-CSF) was inhibited by the ectopic expression of the AML1–MTG8 fusion protein in L-G murine myeloid progenitor cells. We show here that overexpression of normal AML1 proteins reverses this inhibition and restores the competence to differentiate. Immunoprecipitation analysis shows that p300 and CREB-binding protein (CBP) interact with AML1. The C-terminal region of AML1 is responsible for the induction of cell differentiation and for the interaction with p300. Overexpression of p300 stimulates AML1-dependent transcription and the induction of cell differentiation. These results suggest that p300 plays critical roles in AML1-dependent transcription during the differentiation of myeloid cells. Thus, AML1 and its associated factors p300 and CBFβ, all of which are targets of chromosomal rearrangements in human leukemia, function cooperatively in the differentiation of myeloid cells.**

*Keywords*: AML1/cell differentiation/leukemia/p300/transcriptional regulation

## Introduction

Chromosome translocations associated with human leukemia frequently involve genes that code for a variety of transcriptional factors implicated in the regulation of normal hematopoiesis (Rabbits, 1994). The AML1/CBFβ transcription factor complex is the most frequent target of these translocations (Look, 1997). The *AML1* gene (on chromosome 21) was identified through its involvement in the t(8;21) translocation, which occurs in ~40% of acute myeloid leukemia (AML) with the M2 French–American–British subtype (Miyoshi *et al*., 1991). In this translocation, the *AML1* gene becomes juxtaposed to a gene which encodes a zinc finger-containing protein, MTG8 (also known as ETO and CDR), resulting in the expression of AML1–MTG8 chimeric proteins (Erickson *et al*., 1992; Nisson *et al*, 1992; Kozu *et al*., 1993; Miyoshi *et al*, 1993). In addition, the t(12;21) translocation results in the formation of the TEL–AML1 chimeric product through the fusion of the *AML1* gene with the *TEL* gene, which encodes a member of the Ets family of transcription factors. The resultant chimeric transcripts are detected in pediatric B-cell progenitor acute lymphoblastic leukemia, the most common form of leukemia seen in children (Golub *et al*., 1995; Romana *et al*., 1995). Furthermore, AML1-containing fusion products are formed by a t(3;21) translocation which occurs in myelodysplastic syndrome (MDS) and in the blast crisis phase of chronic myelogenous leukemia (Nucifora *et al*., 1993, 1994; Mitani *et al*., 1994). Moreover, CBFβ/PEBP2β, which forms a heterodimer with AML1, is also the target of another leukemia-associated chromosomal rearrangement, inv(16), which often observed in AML-M4Eo (Liu *et al*., 1993).

The AML1 family of transcription factors (Levanon *et al*., 1994) form heterodimeric complexes with CBFβ (also known as PEBP2β) and regulate the transcription of target genes by binding to the DNA sequence TGT/cGGT (Ogawa *et al*., 1993a,b; Wang *et al*., 1993). An 128 amino acid region that is highly homologous to the *Drosophila* segmentation gene *Runt* (Kania *et al*., 1990) is required for heterodimerization with CBFβ/PEBP2β as well as for DNA binding, and this region has been called the runt homology domain (Daga *et al*., 1992; Kagoshima *et al*., 1993; Meyers *et al*., 1993). At least three forms of AML1 protein are produced by alternative splicing. The AML1b isoform (453 amino acids) and the AML1c isoform (480 amino acids) contain the runt homology domain and a C-terminal transcriptional activation domain, whereas the AML1a isoform (250 amino acids) contains the runt homology domain but not the transcriptional activation domain (Miyoshi *et al*., 1995). Possible transcriptional targets include the T-cell antigen receptors (TCRs) (Prossor *et al*., 1992; Redondo *et al*., 1992; Wang *et al*., 1993), the colony-stimulating factor 1 (CSF1/M-CSF) receptor (Zhang *et al*., 1994), myeloperoxidase, neutrophil elastase (Nuchprayoon *et al*., 1994), interleukin-3 (IL-3) (Shomaker *et al*., 1990), granulocyte–macrophage colony-stimulating factor (GM-CSF) (Frank *et al*., 1995; Takahashi *et al*., 1995) and granzyme B (Wargnier *et al*., 1995). Targeted disruption has demonstrated that both AML1 and CBFβ/PEBP2β are essential for all lineages of definitive hematopoiesis in mouse fetal liver (Okuda *et al*., 1996; Sasaki *et al*., 1996; Wang *et al*., 1996a,b; Niki *et al*., 1997).

p300 and CBP are functionally conserved transcriptional coactivators (Arany *et al*., 1995; Lundblad *et al*., 1995) and initially were identified as cellular proteins which bind to the adenovirus-E1a oncoprotein and transcription factor CREB, respectively (Chrivia *et al*., 1993; Eckner *et al*., 1994). p300 and CBP function as transcriptional

coactivators for a large number of transcription factors including CREB (Kwok *et al*., 1994), AP-1 (Arias *et al*., 1994; Bannister and Kouzarides, 1995), c-Myb (Dai *et al*., 1996; Oelgeschlager *et al*., 1996), bHLH proteins (Eckner *et al*., 1996; Yuan *et al*., 1996), nuclear hormone receptors (Chakravarti *et al*., 1996; Kamei *et al*., 1996), STATs (Bhattacharya *et al*., 1996; J.Zhang *et al*., 1996), NF-κB (Perkins *et al*., 1997) and p53 (Avantagiatti *et al*., 1997; Gu *et al*., 1997; Lill *et al*., 1997), and also interact with a basal transcription factor TFIIB (Kwok *et al*., 1994). In addition, p300 and CBP bind to a histone acetyltransferase, P/CAF (Yang *et al*., 1996), and show intrinsic acetyltransferase activity (Ogryzko *et al*., 1996). Thus, p300 and CBP act as multifunctional adaptor proteins to regulate transcription through acetylation of chromatin and recruitment of basal transcription factors. The *CBP* gene recently was shown to be fused to the *MOZ* gene and the *MLL* gene by translocations t(8;16) and t(11;16), respectively, in AML and myelodysplastic syndrome (MDS) (Borrow *et al*., 1996; Rowly *et al*., 1997; Satake *et al*., 1997; Sobulo *et al*., 1997; Taki *et al*., 1997). More recently, we found that p300 is also a target of chromosome translocations and that it makes a fusion protein with MLL through the t(11;22) translocation in AML (Ida *et al*., 1997).

The AML1–MTG8 leukemic fusion protein induces granulocyte colony-stimulating factor (G-CSF)-dependent proliferation of murine hematopoietic precursor L-G cells and inhibits their differentiation to mature neutrophils (Kitabayashi *et al*., 1998). We show here that overexpression of AML1 overcomes the effect of AML1–MTG8. This finding provides a unique system to dissect the functional domains of AML1 and has allowed us to demonstrate a specific interaction and functional cooperation between AML1 and p300. The results, together with the fact that p300 is the target of a leukemia-associated chromosomal translocation, suggest that the AML1–p300 complex may play a crucial role in hematopoiesis.

## Results

### *Overexpression of AML1b overcomes AML1–MTG8-mediated inhibition of myeloid cell differentiation*

Murine L-G is an IL-3-dependent myeloid precursor cell line that can be induced to differentiate into mature neutrophils in response to G-CSF (Kinashi *et al*., 1991). Recently, we found that ectopic expression of AML1–MTG8 in L-G cells inhibits G-CSF-dependent differentiation to neutrophils (Kitabayashi *et al*., 1998). It has been shown that AML1–MTG8 interferes with the function of AML1 (Meyers *et al.*, 1995). This finding suggests that the AML1–MTG8-mediated inhibition of cell differentiation might result from inhibition of AML1 function. If this were the case, then a high level of expression of functional AML1 protein should overcome the inhibition of cell differentiation by AML1–MTG8. To examine this hypothesis, the L-G cells expressing AML1–MTG8 were infected further with retroviruses encoding AML1a or AML1b (Figure 1). Immunoprecipitation analysis showed that AML1b and AML1a proteins were strongly expressed in the respective infectants (Figure 2A). The levels of

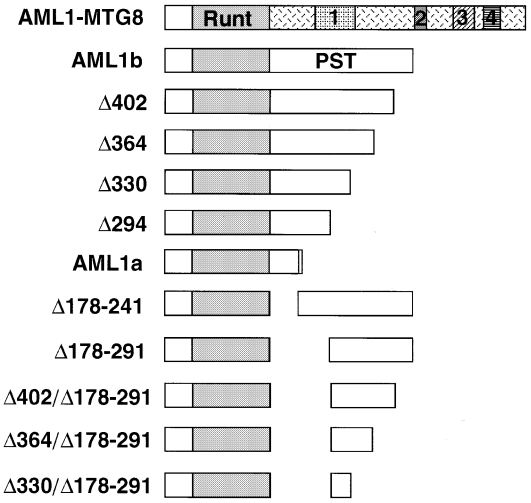


**Fig. 1.** Schematic representation of the structure of AML1–MTG8 and AML1 deletion mutants. The runt homology domain (Runt), the proline-, serine- and threonine-rich regions (PST), and the Nervy homology regions (1–4) are indicated. AML1–MTG8 retains the runt homology domain of AML1 and almost all of the region of MTG8. AML1–MTG8 and AML1a lack the C-terminal transactivation domain (PST) which is present in AML1b.

AML1b and AML1a were estimated to be ~8-fold and 10-fold higher, respectively, than that of AML1–MTG8. When L-G infectants, which overexpressed both AML1b and AML1–MTG8 (AM/AML1b), were cultured in the presence of G-CSF, they proliferated slowly for 2–4 days, but thereafter their growth rate gradually decreased and they stopped growing 4 days after exposure to G-CSF (Figure 2B). Eight days after exposure to G-CSF, they showed morphological differentiation to mature neutrophils with segmented nuclei, like the parental L-G cells (Figure 2D). On the other hand, control cells which expressed only AML1–MTG8 (AM/vector) and cells which expressed both AML1a and AML1–MTG8 (AM/AML1a) proliferated exponentially for at least 11 days without maturation (Figure 2B–D). These results indicate that overexpression of AML1b suppresses the effects of AML1–MTG8 on the response of L-G cells to G-CSF. In other words, AML1b has the ability to restore the competence to differentiate in response to G-CSF. This ability would appear to depend on the C-terminal region of AML1b, since AML1a, which differs from AML1b only in this region, did not induce cell differentiation.

### *Domains of AML1 responsible for differentiation of L-G cells*

To define the region that is essential for AML1b-mediated induction of cell differentiation, a series of deletion mutants (see Figure 1) was constructed. The L-G cells which expressed AML1–MTG8 as described above were infected further with retroviruses encoding the mutants of AML1. Western blot analysis indicated that all of the infectants effectively expressed the expected sizes of the respective proteins (Figure 3). The infectants were cultured in the presence of G-CSF. Representative results are shown in Figure 4A–C, and growth data 11 days after exposure to G-CSF are summarized in Figure 4D. C-terminal deletion analysis indicated that the suppression of cell growth by AML1b in response to G-CSF could be reduced

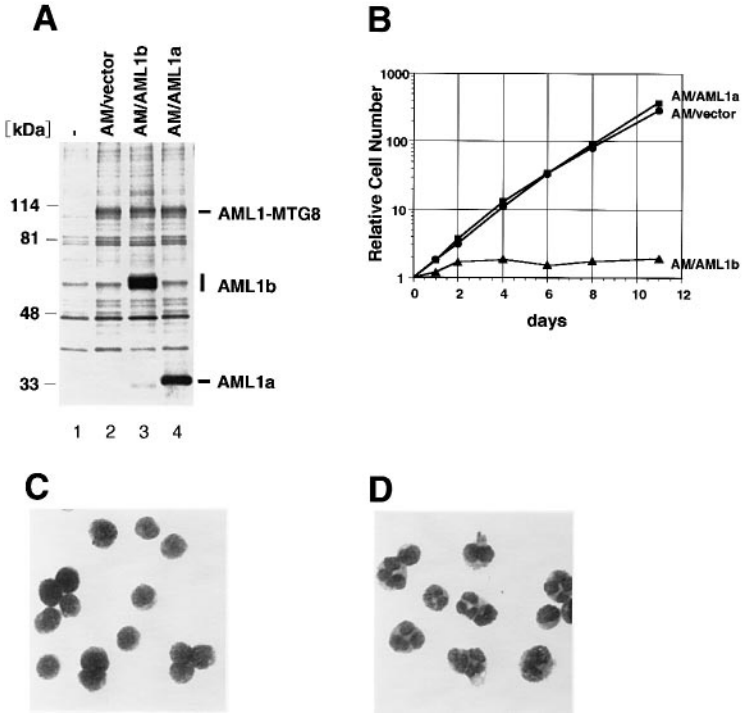


**Fig. 2.** Overexpression of AML1b reverses the effects of AML1–MTG8. (**A**) Immunoprecipitaion of AML1–MTG8, AML1b and AML1a. L-G cells, which expressed HA-AML1–MTG8, were infected further with an LXSH-retrovirus encoding HA-AML1a or HA-AMLb. They were then labeled with [$^{35}$S]methionine, and immunoprecipitations were performed using the anti-HA monoclonal antibody 12CA5. The immunoprecipitates were subjected to SDS–10% polyacrylamide gel electrophoresis. The proteins were visualized using BAS2000. The positions of the bands corresponding to AML1a, AML1b and AML1–MTG8 are indicated on the right. (**B**) Growth curve of the L-G infectants in response to G-CSF. The L-G cells which express AML1–MTG8 (AM) and AML1 proteins were cultured in the presence of 10 ng/ml G-CSF. The relative numbers of viable cells are indicated. (C and D) Morphology of the cells. The L-G infectants which express AML1–MTG8 (**C**) or both AML1–MTG8 and AML1b (**D**) were exposed to G-CSF for 8 days and stained with May–Gruenwald's and Giemsa's solutions.

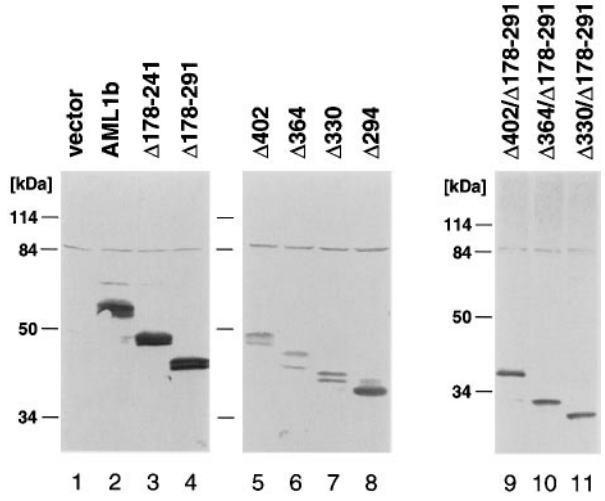


**Fig. 3.** Expression of AML1 mutants. Lysates of L-G infectants which express mutants of HA-AML1 were separated on SDS–10% (lanes 1–8) or 12% (lanes 9–11) polyacrylamide gels and analyzed by immunoblotting using anti-HA antibody.

gradually (but not abolished) by increasing the extent of the deletion from the C-terminal end to positions 402, 364 and 330 (Figure 4A). A further deletion to amino acid 294 completely abolished the ability to induce cell differentiation. Internal deletion of amino acids 178–241 and 178–291 did not strongly affect the ability to suppress G-CSF-dependent proliferation of the L-G infectants (Figure 4B). However, a mutant which lacked both the internal region (178–291) and the C-terminal region (330–451) did not suppress G-CSF-dependent cell proliferation. On the other hand, mutant proteins which contained C-terminal regions beyond amino acid 402 (Δ402/Δ178–291) showed significant activities (Figure 4C). Thus, the region between amino acid 294 and the C-terminus is important for full suppression of the G-CSF-dependent cell proliferation.

### *Differentiation-inducing activity of AML1b correlates with its ability to interact with p300*

p300 and CBP are transcriptional coactivators and are known to interact with a variety of transcriptional factors. Recently, we and others found that both the *p300* and *CBP* genes are disrupted and fused to other genes by chromosome translocation such as t(8;16), t(11;16) and t(11;22) in AML and MDS (Borrow *et al.*, 1996; Ida *et al.*, 1997; Rowly *et al.*, 1997; Satake *et al.*, 1997; Sobulo *et al.*, 1997; Taki *et al.*, 1997). Thus, p300/CBP is involved in leukemogenesis and possibly in the differentiation of myeloid cells, as is the case for AML1 and CBFβ. These findings led us to propose that p300/CBP may act as a coactivator of AML1. To examine this hypothesis, the interaction of p300 with AML1 was analyzed by immunoprecipitation. Cell lysates were prepared from L-G cells infected with retroviruses encoding p300 and hemagglutinin (HA)-tagged AML1b, and immunoprecipitation was performed with AML1-specific and HA-specific antibodies. The immunoprecipitates were separated on SDS–5% PAGE and analyzed by Western

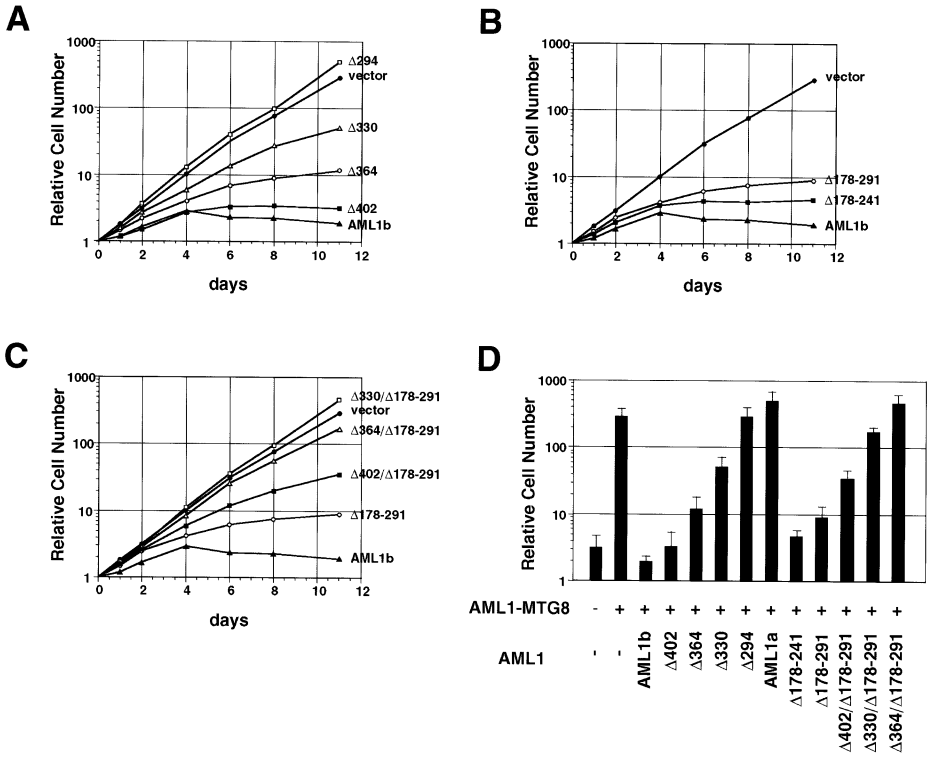


**Fig. 4.** Growth curve of the L-G infectants in response to G-CSF. (**A**–**C**) The L-G cells which express both AML1–MTG8 (AM) and AML1 proteins were cultured in the presence of 10 ng/ml G-CSF. The relative numbers of viable cells are shown. (**D**) The relative numbers of viable cells 11 days after exposure to G-CSF are expressed as bars with standard errors.

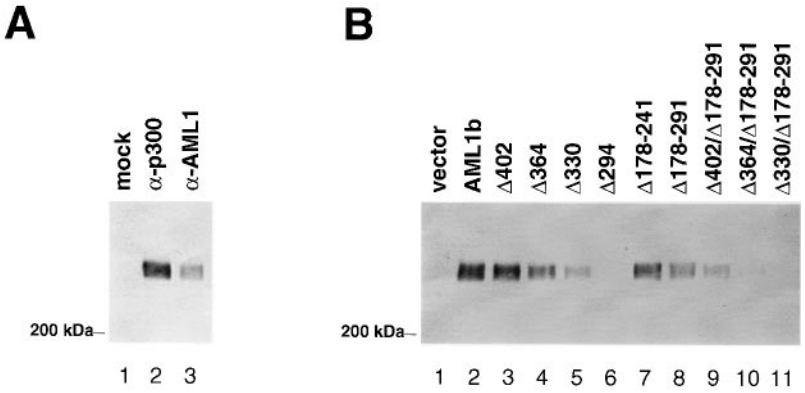


**Fig. 5.** Co-immunoprecipitation of p300 with AML1. (**A**) Lysates of L-G infectants which express both p300 and AML1b were immunoprecipitated with anti-p300 monoclonal antibody, anti-AML1 polyclonal antibody or control rabbit IgG. The immunoprecipitates were separated by SDS–5% PAGE and analyzed by immunoblotting using the anti-p300 monoclonal antibody. (**B**) Lysates of L-G infectants, which express both p300 and mutants of HA-AML1, were immunoprecipitated with anti-HA antibody and analyzed by immunoblotting using the anti-p300 monoclonal antibody.

blotting using p300-specific antibodies. p300 was detected in immunoprecipitates using either AML1-specific antibody (Figure 5A) or HA-specific antibody (Figure 5B).

To test the interaction of endogenous AML1 with p300 and CBP, cell lysates were prepared from uninfected L-G cells. Western blot analysis with p300-specific or CBP-specific antibodies showed that an immunoprecipitate obtained with AML1-specific antibody contained both p300 and CBP, whereas an immunoprecipitate obtained with control IgG contained neither p300 nor CBP (Figure 6). The interaction of AML1 with p300 and CBP could also be demonstrated in other normal myeloid precursor cell lines such as L-GM and 32Dcl3 and a myeloid leukemia cell line HL-60 (Figure 6). These results suggest that AML1 forms complexes with p300 and CBP *in vivo*.

To determine the region of AML1b which is responsible for the interaction with p300, a series of AML1 mutants were expressed in L-G cells and analyzed for their ability to complex with p300. Immunoprecipitation followed by Western blot analysis revealed that complex formation with p300 was reduced gradually (but was not abolish) by increasing the size of the deletion of the C-terminal region of AML1b up to amino acid 330 (Figure 5B). No interaction with p300 was detected when the deletion extended as far as amino acid 294. Internal deletions of amino acids 178–241 or 178–291 decreased, but did not abolish, the ability of AML1b to interact with p300. Deletion of both C-terminal and internal regions (Δ402/Δ178–291, Δ364/Δ178–291, Δ330/Δ178–291) further reduced the interaction with p300. Thus, the region com-

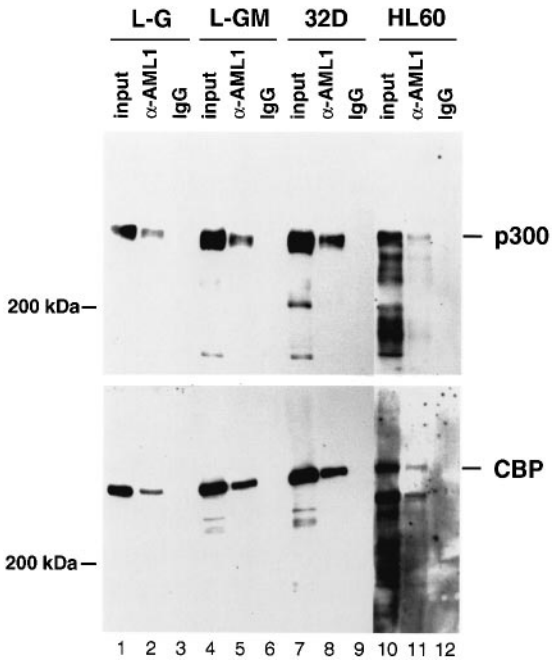


**Fig. 6.** Co-immunoprecipitation of endogenous p300 and CBP with AML1. Lysates of L-G, L-GM, 32Dcl3 and HL60 cells were immunoprecipitated with anti-AML1 polyclonal antibody or control rabbit IgG. The immunoprecipitates and 0.25% of lysates (input) were separated by SDS–5% PAGE and analyzed by immunoblotting using the anti-p300 (upper panel) or anti-CBP (lower panel) polyclonal antibodies.

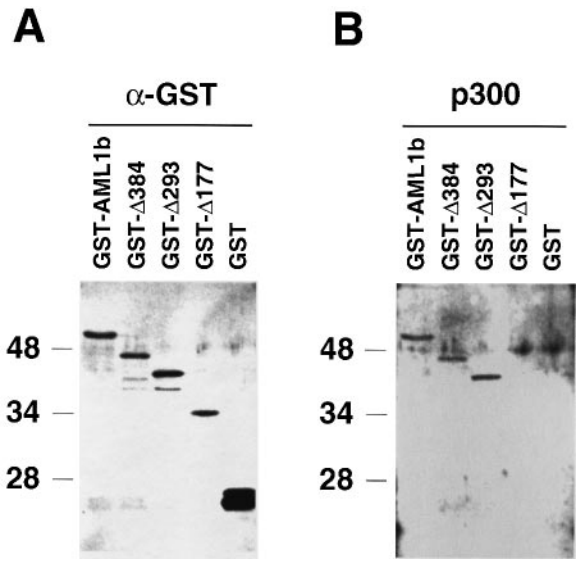


**Fig. 7.** Far Western analysis of the interaction between AML1 and p300. GST–AML1 fusion proteins were separated on SDS–15% PAGE and transferred to a nitrocellulose filter. The filter was probed with anti-GST antibody (**A**) or with purified p300 and anti-p300 monoclonal antibody RW128 (**B**).

prised between amino acid 178 and the C-terminus is critical for the interaction with p300. These results, taken together with the data on the differentiation of L-G cells, suggest that the ability of AML1b to induce differentiation of L-G cells is dependent on its association with p300.

To determine whether AML1 directly interacts with p300, Far Western analysis was conducted using a series of deletion mutants of the recombinant GST–AML1 fusion protein and the p300 protein which had been purified to homogeneity from human 293 cells (Kitabayashi *et al*., 1995). As shown in Figure 7, p300 could bind to GST–AML1b containing most of the AML1b sequence, suggesting a direct interaction between AML1 and p300. Deletion of GST–AML1 from the C-terminus to amino acid residue 294 barely affected binding to p300, but a deletion up to residue 177 abolished binding. These results indicate that the region comprised between amino acids 178 and 294 is important for binding to p300 *in vitro*. The results of FarWestern analysis are similar to, but do not completely coincide with, the results of the immunoprecipitation analysis. These differences suggest that the interaction between AML1 and p300 might be controlled by other factors such as modification by phosphorylation and/or binding of other co-factors such as CBFβ and ALY *in vivo*.

### *N-terminal region of p300 is required for binding of AML1*

To identify the region of p300 responsible for interaction with AML1, a series of deletion mutants of p300 (Figure 8A) were expressed together with AML1b using a retrovirus vector, and the cell extracts were subjected to co-immunoprecipitation analysis. The deletion mutants of p300 were strongly expressed in the respective transfectants (Figure 8B). As shown in Figure 8C, co-precipitation of p300 with AML1b was not seriously affected by deletion of the C-terminal region of p300 up to amino acid 1304. On the other hand, deletion of an N-terminal region (amino acids 142–958) abolished the interaction between p300 and AML1. These results indicate that the N-terminal region of p300 between amino acids 142 and 958 is required for complex formation with AML1b.

### *p300 stimulates AML1-dependent transcription and AML1-induced cell differentiation*

To examine whether p300 acts as a coactivator of AML1, p300 and AML1b were co-transfected with a reporter plasmid carrying the MPO promoter which contains two AML1-binding sites. Co-transfection of p300 together with AML1b significantly activated the MPO promoter in a dose-dependent manner (Figure 9A). AML1 alone or p300 alone did not transactivate the MPO promoter, suggesting cooperative action of AML1 and p300. In addition, the activation of the MPO promoter was not induced by a mutant of p300 lacking the N-terminal region required for interaction with AML1b (Figure 9B). Taken together, these results suggest that p300 functions as a coactivator of AML1 to activate the MPO promoter.

We isolated an L-G subline (L-G/R) which is resistant to G-CSF. As shown in Figure 10B, the L-G/R cells proliferated exponentially in response to G-CSF. To test effects of AML1b and p300 on G-CSF-dependent growth of the L-G/R cells, AML1b and/or p300 were introduced into the cells using retrovirus vectors, and the infectants were cultured in the presence of G-CSF. While overexpression of AML1b resulted in a significant decrease in the growth rate of L-G/R cells, p300 did not affect G-CSF-dependent proliferation (Figure 10B). Overexpression of p300 together with AML1b further suppressed G-CSF-dependent cell proliferation. AML1b and p300 did not affect IL-3-dependent proliferation of L-G/R cells (data

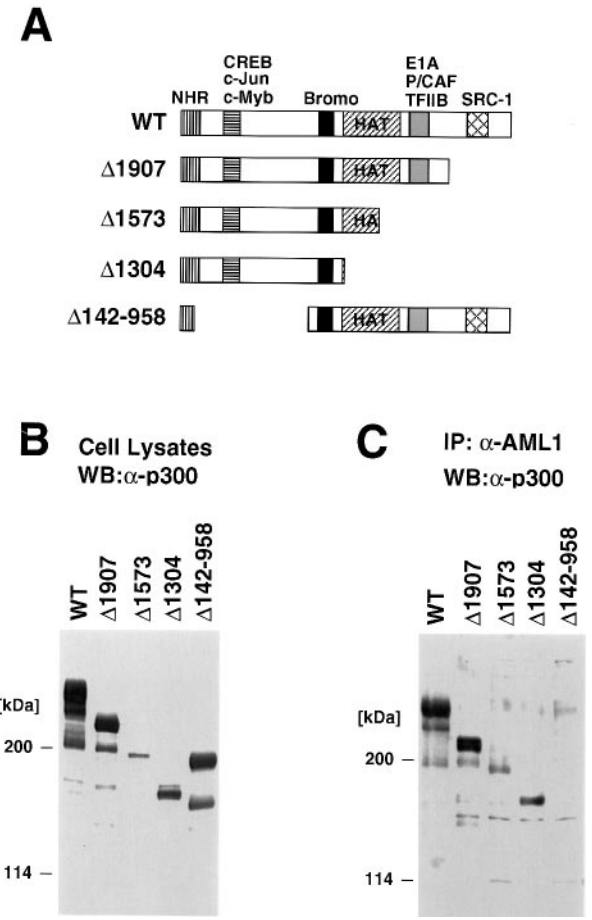


**Fig. 8.** The N-terminal region of p300 is required for interaction with AML1. (**A**) Schematic representation of the structure of p300 deletion mutants. The histone acetyltransferase domain (HAT), the bromodomain (Bromo) and the regions which bind to nuclear hormone receptors (NHR), CREB, c-Jun, c-Myb, E1A, P/CAF, TFIIB and SRC-1 are indicated. (**B**) Lysates of L-G infectants which express both AML1b and mutants of p300 were separated by SDS–7% PAGE and subjected to immunoblot analysis using the anti-p300 polyclonal antibody. (**C**) Lysates of L-G infectants which express both AML1b and mutants of p300 were immunoprecipitated with anti-AML1 antibody and subjected to immunoblot analysis using the anti-p300 polyclonal antibody.

not shown). Thus, p300 and AML1 function cooperatively in suppression of the G-CSF-dependent cell proliferation.

To determine the region of p300 which is responsible for the cooperative function with AML1, a series of p300 mutants were expressed together with AML1b in L-G cells and these cells were cultured in the presence of G-CSF. Representative growth curves are shown in Figure 10C and results are summarized in Figure 10D. Internal deletion of the region between amino acids 142 and 958 which is required for interaction with AML1 almost completely abolished the ability to suppress G-CSF-dependent proliferation of the L-G infectants. The suppression of cell growth by p300 in response to G-CSF was reduced by the deletion from the C-terminal end to position 1907. A further deletion to amino acid 1573 completely abolished the ability to suppress cell proliferation. These results suggest that the cooperative function of AML1 and p300 required the N-terminal region of p300, which is needed for their physical interaction, as well as the C-terminal region of p300, which is responsible

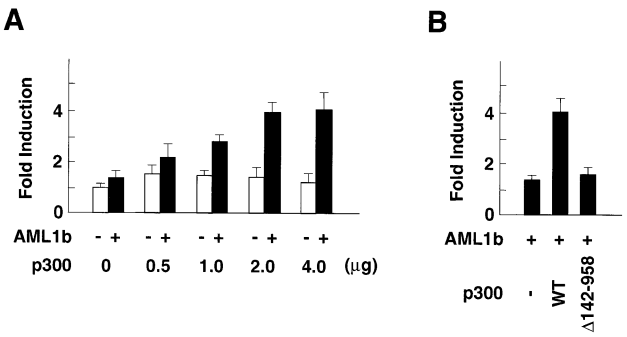


**Fig. 9.** Activation of AML1-dependent transcription by p300. (**A** and **B**) P19 cells were co-transfected with 0.5 μg of MPO-luciferase, 2.0 μg of either the pLNSX (–) or pLNSX-AML1b (+) vector, the indicated amounts (μg) of pACT-p300 and 0.1 μg of pRL in a 6 cm plate. Results represent the average value for relative luciferase activity from three experiments which were normalized using the activity of the enzyme from pRL as an internal control.

for the intrinsic histone acetyltransferase acitivity and the interaction with histone acetyltransferases such as P/CAF and SRC-1 (see Figure 8A).

## Discussion

We previously found that expression of the AML1–MTG8 leukemic fusion protein in L-G myeloid precursor cells blocks G-CSF-dependent maturation into neutrophils (Kitabayashi *et al*., 1998). We show here that overexpression of AML1 reverses this effect and induces differentiation of L-G transformants which express AML1–MTG8. Using this system, we also demonstrated that the p300 transcriptional coactivator interacts with the C-terminal region of AML1, the region found to be responsible for stimulation of cell differentiation and transcriptional activation. In addition, AML1 and p300 function cooperatively in activation of transcription and in stimulation of differentiation of L-G cells. Thus, p300 probably plays an important role in AML1-dependent transcriptional regulation during the differentiation of myeloid cells.

### *AML1/CBFβ/p300 (CBP) complex: the target of leukemia-associated translocations*

The *AML1* gene is the most frequent target of chromosome translocations in human leukemia (Look, 1997). CBFβ, the heterodimeric partner of AML1, is also mutated in inv(16) which is associated with AML (Liu *et al*., 1993). These findings suggest that leukemic transformation may be the result of altered expression of genes regulated by AML1. We found that CBP as well as p300 could be detected in immunoprecipitates of AML1 (Figure 6). These findings suggest that p300/CBP may play critical roles in the function of AML1 and in hematopoiesis, and that an altered function of p300/CBP could change the expression of genes regulated by AML1. In accordance with this concept, we and others recently found that both *CBP* and *p300* genes are disrupted and fused in-frame to other genes such as *MOZ* and *MLL* by chromosomal translocations in AML and MDS (Borrow *et al*., 1996; Ida *et al*., 1997; Rowly *et al*., 1997; Satake *et al*., 1997; Sobulo *et al*., 1997; Taki *et al*., 1997). Thus, AML1 and its associated factors p300/CBP and CBFβ are all targets of chromosomal rearrangements in human leukemia. These findings suggest

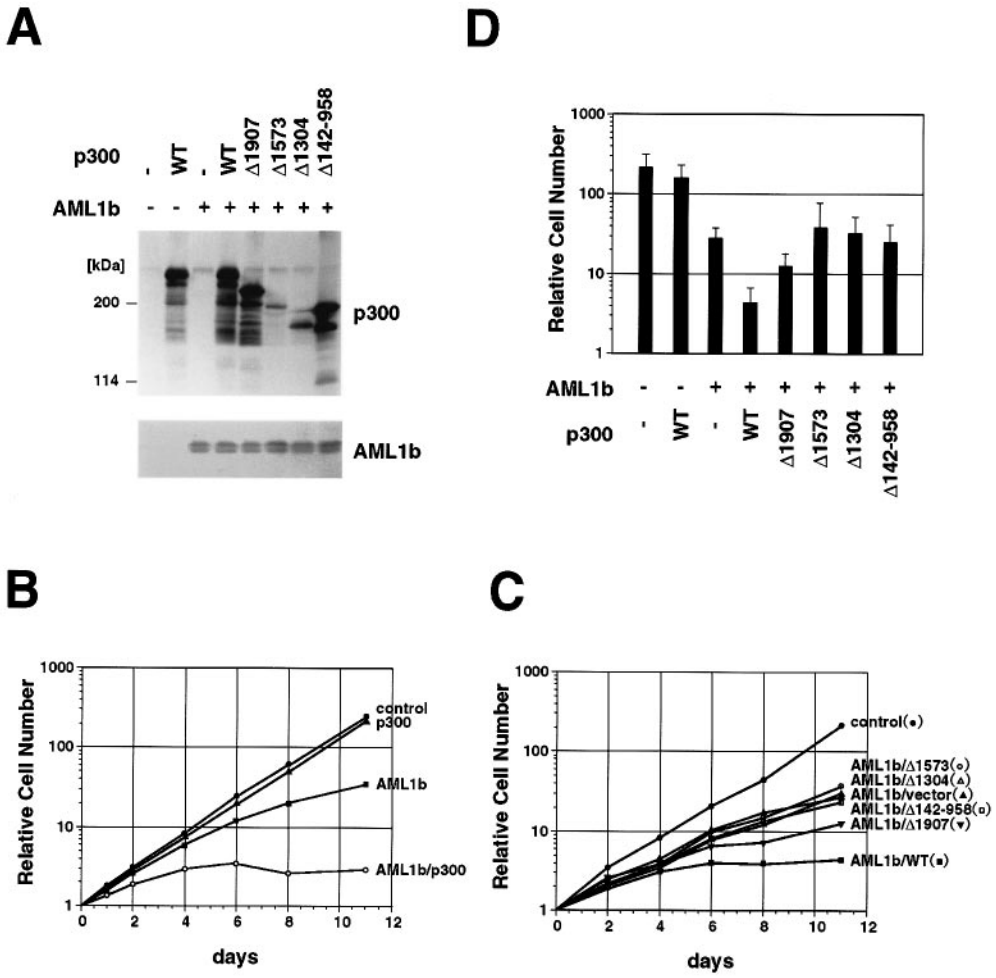


**Fig. 10.** p300 stimulates the ability of AML1b to suppress G-CSF-dependent proliferation of L-G/R cells. (**A**) Expression of AML1 and p300 mutants. Lysates of L-G infectants which were infected with LXSH-HA-AML1b and LNCX-wild type (WT) or mutants of p300 were separated on SDS–6% (for p300) or 10% (for AML1) polyacrylamide gels and analyzed by immunoblotting using anti-p300 or anti-AML1 antibodies. (**B** and **C**) The infectants were cultured in the presence of 10 ng/ml G-CSF. The relative numbers of viable cells are shown. (**D**) The relative numbers of viable cells 11 days after exposure to G-CSF are expressed as bars with standard errors.

that the p300/CBP-containing chimeric products as well as CBFβ fusions might affect AML1-dependent transcriptional regulation of genes involved in hematopoiesis, resulting in the promotion of leukemogenesis. It will be interesting to analyze whether p300/CBP-containing chimeric products affect AML1-dependent transcription and the differentiation of myeloid cells.

### *Cooperation of AML1 with other factors*

It has been suggested previously that AML1 acts to facilitate the action of other adjacent transcription factors. AML1 and c-Myb were shown synergistically to activate the TCR δ chain gene, the myeloperoxidase gene and the SL3 retroviral long terminal repeat (Hernandez-Munain and Krangel, 1994, 1995; Zaiman and Lenz, 1996; Britos-Bray and Friedman, 1997). Biochemical analysis indicated that AML1 and c-Myb do not bind DNA cooperatively, and that c-Myb does not interact directly with AML1 bound to DNA (Hernandez-Munain and Krangel, 1995; Zaiman and Lenz, 1996). These findings suggest that AML1 and c-Myb may require additional cooperating factor(s) to mediate synergy between these factors (Hernandez-Munain and Krangel, 1995). The C-terminal transactivation domain of AML1 is sufficient for cooperation with c-Myb (Britos-Bray and Friedman, 1997). The present results indicate that the C-terminal domain of AML1 is also required for interaction with p300 and for G-CSF-dependent induction of cell differentiation. It has been reported that p300/CBP can interact with c-Myb (Dai *et al*., 1996; Oelgeschlager *et al*., 1996). These results suggest that the functional synergy of AML1 and c-Myb may be mediated by p300/CBP. AML1, p300 (CBP) and c-Myb may form a multimeric complex on the promoter to recruit components of the transcriptional machinery and/or acetylate chromatin, resulting in strong activation of transcription (Figure 11).

AML1 also synergistically activates the M-CSF receptor promoter in the presence of C/EBP (D.E.Zhang *et al*., 1996). Although a physical interaction between AML1 and C/EBP has been observed, no cooperativity in the DNA binding of AML1 and C/EBP was detected at their adjacent sites on the M-CSF promoter (D.E.Zhang *et al*., 1996). Recently, it was reported that p300 interacts with, and functions as a coactivator for, C/EBP (Mink *et al*., 1997). These findings suggest that p300/CBP coactivators may mediate functional synergy between AML1 and

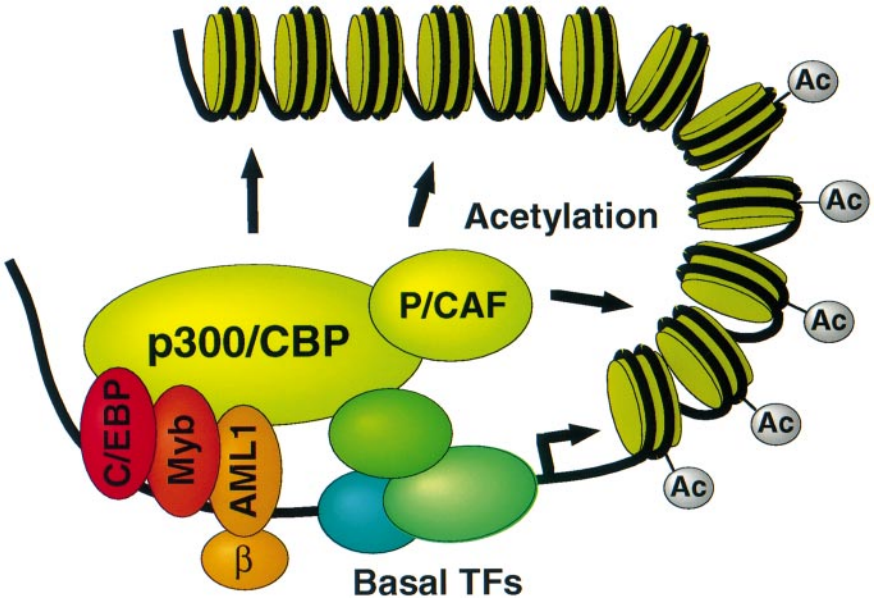


**Fig. 11.** Model for the action of AML1 and p300/CBP in the activation of transcription. AML1 forms a heterotrimer complex with CBFβ/PEBP2β (β). The AML1/β complex, c-Myb and C/EBP bind to the respective DNA sequences of the MPO promoter and form a multimeric complex with p300/CBP and P/CAF to activate *MPO* gene expression though the recruitment of the basal transcription factors (TFs) and chromatin acetylation.

C/EBP by enhancing their association. Indeed, it is quite possible that p300/CBP forms a multicomplex with more than one transcription factor to activate the transcription of their target genes (Figure 11).

We found that the MPO promoter/enhancer reporter is activated by co-transfection of p300 together with AML1 (Figure 9) but that the TCR β chain enhancer reporter is not strongly activated by co-transfection of p300 (data not shown). In addition, AML1 did not strongly activate the MPO enhancer without co-transfection of c-Myb (Britos-Bray and Friedman, 1997), but it could activate the TCR β chain enhancer without co-transfection of other factors (Bae *et al*., 1994). These results suggest that different sets of coactivators may be involved in the AML1-mediated transcription of these genes depending on the cell types and/or the promoter contexts. It was reported recently that ALY can interact with AML1 and LEF-1, and that it functions as a context-dependent coactivator at the TCR α enhancer (Bruhn *et al*., 1997). Our results suggest that the p300–AML1 complex may play an important role in the transcriptional regulation of genes implicated in myeloid cell differentiation. Further studies of the effects of p300/CBP and other factors, including ALY, c-Myb and C/EBP, on the AML1-dependent transcription of various promoters should contribute to our understanding of the mechanism of transcriptional regulation by AML1.

### *AML1 family and p300/CBP*

The three members of the *AML1* gene family, *AML1* (*CBFa2/PEBP2aB*), *AML2* (*CBFa3/PEBP2aC*) and *AML3* (*CBFa1/PEBP2aA*), have been isolated (Levanon *et al*., 1994). Since the C-terminal regions of the AML1 family members are conserved, p300/CBP may also interact with AML2 and AML3. AML3-deficient mice are defective in osteoblast differentiation and bone development, and show various skeletal abnormalities including those of the sternum, xiphoid process and clavicle (Komori *et al*., 1997; Otto *et al*., 1997). Mutations in AML3 have been reported in human cleidocranial dysplasia syndrome which is characterized by hypoplasia/aplasia of clavicles, patent fontanelles, supernumerary teeth, short stature and other changes in skeletal patterning and growth (Mundlos *et al*., 1997). On the other hand, the *CBP* gene is reported to be mutated in Rubinstein–Taybi syndrome which is a well-defined syndrome characterized by multiple congenital malformation and mental retardation as its main clinical features (Petrij *et al*., 1995). Furthermore, mice which heterozygously lack CBP show various skeletal abnormalities including delayed ossification, large anterior fontanel and abnormal ossification in the sternum, xiphoid process and axial bone (Tanaka *et al*., 1997). Thus, some of the features of the malformations of Rubinstein–Taybi syndrome and of CBP-deficient mice resemble those of cleidocranial dysplasia syndrome and AML3-deficient mice, with the exception of mental retardation which is not a feature of cleidocranial dysplasia syndrome. These similarities of CBP-deficient and AML3-deficient mice suggest that CBP may also function as a coactivator for AML3, and that mutations in CBP could change the expression of genes regulated by AML3. It will be interesting to analyze the interaction of CBP and p300 with other AML1 family members and study the expression of genes regulated by AML1 family members.

## Materials and methods

### *Cells and retroviruses*

L-G and L-GM cells (Kinashi *et al*., 1991) were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS), recombinant mouse IL-3 (0.1 ng/ml) (a generous gift from Kirin Brewery) and 50 μM β-mercaptoethanol. BOSC23 (Pear *et al*., 1993) and P19 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. LNSX and LXSH retrovirus vectors (Miller *et al*., 1993) were generously provided by Dr D.Miller. For production of retroviruses, BOSC23 cells were transfected with LNSX- or LXSH-derived retrovirus vectors by calcium phosphate precipitation methods, and culture supernatants were recuperated 48 h after transfection. L-G cells were incubated in the culture supernatant of BOSC23 transfectants for 24 h and then

selected with G418 (1 mg/ml) or hygromycin B (2.5 mg/ml). When L-G cells were exposed to G-CSF, the cells maintained in the presence of IL-3 were washed twice with IL-3-free medium and incubated in medium containing human recombinant G-CSF (2 ng/ml) (a generous gift from Chugai Pharmaceutical Company). Viable cells were counted using a Coulter counter (Coulter Electronics Ltd). For cell morphology, cells were stained with May–Grunwald's solution and Giemsa's solution (Merck).

### *Plasmid construction*

The N-terminal HA-tag was fused to the AML1a, AML1b or AML1–MTG8 cDNA by using the oligonucleotide 5′-CCTAGGCCT CTAGACCATGGCATACCCATACGACGTGCCTGACTACGCCTC-CCGTATCCCCGTAGATGCC-3′ as the upstream primer and 5′-AGACAGTGATGGTCAGAGTG-3′ as the downstream primer in a PCR. The PCR product was digested with *Stu*I and *Hin*dIII, and ligated to the *Hin*dIII site near the N-terminus of AML1a, AML1b and AML1–MTG8. Deletion mutants of AML1 were constructed by ligation of the DNA fragments which were generated by appropriate restriction enzymes and PCR. Human p300 cDNA (Eckner *et al*., 1994) was generously provided by Dr D.M.Livingston. Deletion mutants of p300 were generated by digestion with the appropriate restriction enzymes and linker ligation. The sequences of the above constructs were checked by sequencing.

### *Immunoprecipitations*

Immunoprecipitations were performed as described previously (Kitabayashi *et al*., 1995). Cells were metabolically labeled for 4 h with [$^{35}$S]methionine (50 μCi/ml; Amersham) in methionine-free DMEM. The cells were lysed by incubation at 4°C for 30 min in lysis buffer [20 mM sodium phosphate pH 7.0, 250 mM NaCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM $Na_3VO_4$ and 1 mM phenylmethylsulfonyl fluoride (PMSF)] supplemented with 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin. The lysates were cleared by centrifugation at 30 000 *g* for 30 min at 4°C and the supernatants were saved and stored at –80°C. The cell lysates were incubated with anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim), anti-AML1 polyclonal antibody or anti-p300 monoclonal antibody RW128 (Upstate Biotechnology) on ice for 1 h, followed by addition of protein G–Sepharose beads (Pharmacia) and rotation at 4°C for 2 h. The beads were washed five times with 1 ml of lysis buffer. Proteins were separated on SDS–polyacrylamide gels and visualized by the imaging analyzer BAS2000 (Fuji).

### *Purification of p300*

p300 was purified from 293 cells, which constitutively express E1A proteins, as described previously (Kitabayashi *et al*., 1995). Cell lysates were incubated with E1A-specific monoclonal antibody M73-conjugated beads by rotation at 4°C for 1 h. The beads with absorbed E1A immunocomplexes were packed into a column and washed with 10 vols of lysis buffer. p300 was eluted selectively with a high detergent buffer [50 mM Tris–HCl pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM PMSF and 10% glycerol]. The fraction containing p300 was dialyzed against column buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 10% glycerol) and applied to a Q-Sepharose FF column (Pharmacia), and the column was washed extensively with column buffer. p300 was eluted with column buffer containing 0.3 M NaCl.

### *Western blotting*

Cell lysates or immunoprecipitates were fractionated on SDS–polyacrylamide gels and transferred to membrane filters (Hybond ECL; Amersham) by electroblotting. Filters were blocked in 5% low-fat milk, dissolved in phosphate-buffered saline plus 0.1% Tween-20 (PBST) at room temperature for 2 h or at 4 °C overnight. After extensive washing in PBST, the filters were incubated for 1 h at room temperature with the anti-p300 monoclonal antibody RW128, anti-HA monoclonal antibody 12CA5, anti-p300 or anti-CBP polyclonal antibodies. After further washes in PBST, the filters were incubated with horseradish peroxidase-conjugated secondary antibodies and then washed extensively in PBST. The immunocomplexes were visualized by an ECL detection system (Amersham). The anti-AML1 and the anti-p300 polyclonal antibodies were generated by immunizing rabbits with peptides corresponding to residues 8–24 of human AML1a and residues 2–18 of human p300, respectively, and were affinity purified using antigen-conjugated columns. The anti-CBP polyclonal antibody was from Santa Cruz Biotechnology.

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